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understanding of the basic immunol. of target **antigens** and their resp. antibodies. Of significance, a-CL Abs cross-react with other anionic **phospholipids**. Addnl., the results of these assays led to the realization that high levels of circulating a-PL Abs over long periods are assocd. with a no. of clin. problems now known collectively as the antiphospholipid syndrome.

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ACCESSION NUMBER: 1995:739359 CAPLUS
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AB Liposomes of stable multilamellar type, which previously demonstrated great efficiency in antibiotic transport, were used in this study as transport vehicles of antigenic exts. of Brucella melitensis (HS: complex of lipopolysaccharide/**phospholipids** /outer membrane **proteins**). The incorporation of HS into **pos. charged** liposomes produced a protective effect against exptl. murine brucellosis when they were administered 1 day before or 2 days after **infection**, as the no. of colony-forming units in the spleen was reduced in relation to the untreated control group. The use of HS-free or bound in liposomes with **neg.** net **charge** did not produce a significant effect. Moreover, the incorporation of HS into cationic liposomes eliminated the toxicity of the lipopolysaccharide.

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Protective effect of *Brucella* outer membrane complex-bearing liposomes against experimental murine brucellosis

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Abstract

Liposomes of stable multilamellar type, which previously demonstrated great efficiency in antibiotic transport, were used in this study as transport vehicles of antigenic extracts of *Brucella melitensis* (HS: complex of lipopolysaccharide/phospholipids/outer membrane proteins). The incorporation of HS into positively charged liposomes produced a protective effect against experimental murine brucellosis when they were administered 1 day before or 2 days after infection, as the number of colony-forming units in the spleen was reduced in relation to the untreated control group ($P < 0.01$). On the other hand, the use of HS-free or bound in liposomes with negative net charge did not produce a significant effect. Moreover, the incorporation of HS into cationic liposomes eliminated the toxicity of the lipopolysaccharide.

Keywords: Liposome; *Brucella*; Lipopolysaccharide

1. Introduction

Brucellosis is a zoonosis caused by different species of *Brucella*. Its importance lies in the enormous socio-economic repercussion of the illness, as apart from being a major animal health problem it also constitutes a serious risk to human health, mainly in rural areas. In animals, this illness mainly leads to abortions and reproductive organ disorders in males and females, leading to infertility [1].

Given the widespread need for vaccination in brucellosis control, much research has been done to find a vaccine which provides a high level of resistance without producing negative side effects. Vaccines currently used on livestock generally consist of live attenuated strains of *Brucella* in smooth phase [2,3]. Despite the fact that the use of these vaccines has an acceptable protective effect, there are many disadvantages associated with it, mainly related to residual virulence and post-vaccine antibodies, which interfere in diagnostic tests [4–6]. For this reason, it is important to find new protective systems.

Liposomes may be used as immunological adjuvants to induce or modulate the immunological response to adsorbed or encapsulated antigens. Liposomes hold an advantage over conventional adjuvants in that their toxicity is low, as they are made up of bio-compatible components. Heath et al. [7] showed that their adjuvant power could mainly be related to their efficient capture by macrophages and the stimulus they exert on the immune cellular system. In this respect, liposomes have been prepared

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efficiently with antigens from *Vibrio cholerae* on their surface [8], and from the malaria agent [9], Hepatitis B virus [10] and *Salmonella* [11]. In each case a higher response was obtained than that from the administration of the free antigen.

In this study we have examined the protective effect of antigenic extracts of *B. melitensis* (lipopolysaccharide, phospholipids and external membrane proteins) on liposomes in mice experimentally infected with *Brucella*. The liposome formulation used has been demonstrated previously to be effective in antibiotic transport, being very efficient in the reduction of infection of monocytes experimentally infected with *Brucella* (Vitas et al., unpublished).

2. Materials and methods

2.1. Animals

Female Swiss-Webster mice of 20 ± 2 g weight were used in the experiments. They were kept under conditions of constant temperature and humidity.

2.2. Bacterial strains

B. abortus 2308 and *B. melitensis* 16M strains were used, both in smooth phase. They were kept at −85°C in sterile skimmed milk and (for routine use) were cultivated in trypticase soy agar (TSA). To promote the virulence of the smooth strains, they were inoculated intraperitoneally in mice. After 2 or 3 weeks the animals were killed and the bacteria recovered by culturing a homogenate of the spleen on TSA and incubating it at 37°C for 4 days. The stability of the strains in their smooth phase was checked by exclusion tests [12].

2.3. Extraction method of the HS antigenic complex

To obtain the antigenic hot saline extract (HS), consisting of lipopolysaccharide (LPS), phospholipids and external membrane proteins of *B. melitensis* biotype 1, the method described previously [13,14] was followed. Briefly, 10 g of cells were resuspended in 100 ml of saline solution and heated to 100°C for 15 min. They were then centrifuged at $12\,000 \times g$ for 15 min and the supernatant was collected and dialysed for 2 days at 4°C, with several changes of deionized distilled water. The dialysed material was centrifuged at $100\,000 \times g$ for 5 h, and the sediment (HS) was resuspended in deionized distilled water for later freeze-drying.

2.4. Preparation and characterization of liposomes

The following Sigma Chemical Co. freeze-dried compounds were used to prepare the vesicles: phosphatidylcholine of egg yolk (PC; 60% pure), cholesterol (CHL, 99% pure), diacetylphosphate (DP; 97% pure) and stearylamine (ST; 99% pure). Liposomes of 'stable multilamellar vesicles' type (SPLVs) were prepared according to the technique developed by Vitas et al. (unpublished) with the following composition: PC:CHL:ST (7:3:0.5 M) or PC:CHL:DP (7:3:1 M). This method was based on that of Gruner et al. [15] with some modifications. First, the phospholipid suspension was made in chloroform at a rate of 10 mg/ml. In this phase the HS antigenic extract was dissolved in proportion to phosphatidylcholine (PC) HS/PC 1: 10 (dry weight). After adding the aqueous phase (HEPES buffer: 10 mM HEPES, 145 mM NaCl, 0.02% sodium azide, pH 7.4), the liposome emulsion was obtained by Vortex agitation for 30 s followed by sonication in a bath sonicator (50 W, 2 min at room temperature). The organic phase surplus was eliminated by a vacuum rotovapor at low pressure (200–300 mmHg) for 5 min at a bath temperature of 40°C. N_2 gas was used to eliminate any trace of solvent. The liposomes were resuspended in HEPES buffer by using 5-mm diameter glass beads.

Particle size distribution and zeta potential of the liposomes were determined by Photon Correlation Spectroscopy using a Zetamaster (Malvern Instruments).

The quantification of proteins in solution was done according to the method of Lowry et al. [16], using bovine serum albumin as a standard and in the presence of 1% SDS in the sodium carbonate solution [17].

The amount of LPS was indirectly estimated by determining one of its exclusive markers, KDO, by the thiobarbiturate acid method [18,19]. Pure KDO was used as a standard and a solution standard of D-deoxyribose to correct possible interference from the 2-deoxysugars.

2.5. SDS-PAGE

SDS-PAGE was done on 10.0 × 7.5-cm plates using Laemmli's discontinuous system [20], with 560 mM Tris · HCl (pH 8.8) and 13.5% acrylamide in the separation gel, and 125 mM Tris · HCl (pH 6.8) and 4% acrylamide in the concentration gel. In the cuvettes, 0.5% SDS in 25 mM Tris · HCl and 1 M glycine (pH 8.3) was used. The samples were solubilised at 100°C for 10 min in 62.5 mM Tris · HCl (pH 6.8), 10% glycerol, 0.7 M 2-β-mercaptoethanol, 2% SDS and 0.00125% Bromophenol blue. Electrophoresis was done at a constant intensity of 25 mA. Immediately after electrophoresis, the gels were stained with ammoniacal silver after oxidation with periodate according to the method of Tsai and Frasch [21] with slight modifications [14].

2.6. Prophylactic effect of free or included HS on liposomes with different charges

Thirty-six female Swiss-Webster mice were randomly divided into six homogeneous groups and received (i.v.) a single dose of liposomes (200 μl) of the following composition: liposomes with simple positive net charge (PC/CHL/ST, 7:3:0.5 M) with and without HS (10%); liposomes with negative net charge (PC/CHL/DP, 4:5:1 M) containing or not HS (10%). Another group was inoculated with the HS antigenic extract in free form (dissolved in saline solution), in the same concentration as that included in the liposomes (171 μg/mouse). Finally, a control group did not receive any treatment. After 1 day, all the animals were then infected intraperitoneally with a dose of 6.7×10^4 CFU of *B. abortus* 2308. The mice were killed by cervical dislocation on day 13 after infection. The spleen and liver were aseptically removed from each mouse to determine the number of cfu after plating on TSA.

2.7. Effect of liposomes on the toxicity of the lipopolysaccharide

Homogeneous groups of six mice received a dose (i.v.) of 850 μg of free or included HS extract in liposomes with positive or negative net charge (equivalent to 82.2 μg LPS). To reinforce the entodotoxic shock, 20 mg of galactosamine (HS + galactosamine/1 ml of saline solution) was administered.

2.8. Effect of the administration of HS liposomes before or after infection

Seventy Swiss-Webster female mice (20 ± 2 g) were i.p. infected with a dose of 3.5×10^5 cfu of *B. abortus* 2308. On days −1, +2 or +10 in relation to infection, six groups of ten mice received (i.v.) a dose of liposomes (200 μl) with simple net positive charge (PC/CHL/ST, 7:3:0.5 M), or containing HS antigenic extract on the surface. The control group was infected and not treated. The mice were killed 13 days after infection by cervical dislocation. The spleens were removed under aseptic conditions and, after being homogenized and cultivated on TSA plates, the number of viable *Brucella* cells was determined.

2.9. Statistical analysis

The statistical study was carried out by Student's *t*-test, using the Stat View computer program.

3. Results

Characterization of the included HS antigenic extract in liposomes. Analysis revealed that the HS extract contained 16.7% protein and 0.64% of KDO (LPS marker).

HS was included in SPLV-type liposomes with positive (PC/CHL/ST, 7:3:0.5 M) or negative (PC/CHL/DP, 4:5:1 M) net charge. The presence of HS extract in the liposomes was initially demonstrated by SDS-PAGE. This analysis showed that the protein profile of the liposomes containing HS was similar to that of free HS (not shown). Moreover, the KDO and protein analyses indicated that the amount of HS incorporated was similar in the two types of liposomes, regardless of their composition (Table 1). The presence of the HS antigen on the surface of both types of liposomes was demonstrated by rapid agglutination with serum obtained specifically from mice infected with *B. melitensis*. The physical characteristics of both types of HS liposomes are shown in Table 1.

Table 1
Physicochemical characterization of free HS and HS liposomes

	Charge	Protein (%)	KDO (%)	Polydispersity index	Mean diameter (nm)	Zeta potential (mV)
HS		16.7	0.64	0.578	214.1	ND
HS liposome	Negative [a]	5.7	0.26	0.145	192.9	−19.1
HS liposome	Positive [b]	6.8	0.21	0.296	169.4	+16.9

[a] Liposome composition: PC/CHL/DP 4:5:1 M.
[b] Liposome composition: PC/CHL/ST 7:3:0.5 M.
ND, not determined.

3.1. Prophylactic effect of free or included HS on liposomes with different charges

The results (Table 2) show how the incorporation of the HS antigenic extract in positively charged liposomes produced a protective effect against infection, as the number of cfu in the spleen was reduced by 1.49 log in comparison with the untreated control group ($P < 0.01$). On the other hand, the use of free or included HS in negatively charged liposomes did not produce significant differences in comparison with the untreated control group. Similar infection levels were obtained in the liver (not shown).

3.2. Effect of liposomes on LPS toxicity

The results showed that the incorporation of the HS antigenic extract in liposomes considerably reduced the toxicity of its LPS. The concentration of free HS which killed 50% of the mice under the conditions described in Materials and methods was 850 μg (equivalent to 82.2 μg of LPS). However, when this same concentration of HS was included in the anionic liposomes, mortality dropped to 33.3% and none of the mice died when HS was included in cationic liposomes.

Table 2
Effect of antigenic HS extract-bearing liposomes with different charges on mice infected with *B. abortus* 2308 after administration 1 day before infection

Group	Spleen weight (g)	Log cfu/spleen	Reduction (log)	[d]	[e]
Control	0.287 ± 0.092	6.24 ± 0.17			
Free HS [a]	0.182 ± 0.030	5.84 ± 0.28	0.40	*	
LIPO − [b]	0.208 ± 0.069	5.02 ± 1.07	1.22	**	
LIPO + [c]	0.207 ± 0.044	5.79 ± 0.36	0.45	*	
LIPO − HS	0.248 ± 0.064	5.83 ± 0.23	0.41	*	
LIPO + HS	**0.203 ± 0.061**	**4.75 ± 0.59**	**1.49**	*	**

[a] Amount of HS (free or included in liposomes) received by each mouse: 171 μg.
[b] Liposome composition: PC/CHL/DP 4:5:1 M.
[c] Liposome composition: PC/CHL/ST 7:3:0.5 M.
[d] Statistical analysis: Student's *t*-test. Significant differences in relation to the untreated control group: *, $P < 0.05$; **, $P < 0.01$.
[e] Significant differences compared with the free HS group.

3.3. Prophylactic and therapeutic effect of included HS on liposomes

After selection of cationic liposomes as the best for use with extracts containing LPS, their efficiency in protection against experimental murine *B. abortus* infection was determined. The results in Table 3 show a clear protective effect by the HS complex included in liposomes when these were administered 1 or 2 days after infection. A reduction of 2.86 and 2.22 log, respectively, occurred in these cases when compared with the untreated control group. The sta-

Table 3
Effect of HS liposomes on mice infected with *B. abortus* after administration on days −1, +2 and +10 in relation to the infection

Group	Spleen weight (g)	Log cfu/spleen	Reduction (log) [c]
Control	0.119 ± 0.046	5.90	
Lipo −1 [a]	0.141 ± 0.029	5.34	0.56
Lipo +2	0.130 ± 0.021	5.30	0.60
Lipo +10	0.161 ± 0.019	4.99	0.91 *
Lipo HS −1 [b]	0.206 ± 0.024	3.04	2.86 **
Lipo HS +2	0.304 ± 0.097	3.68	2.22 **
Lipo HS +10	0.293 ± 0.062	6.08	0

[a] Liposome composition: PC/CHL/ST 7:3:0.5 M.
[b] Each mouse recieved 171 μg of HS.
[c] Statistical analysis: Student's *t*-test. Significant differences in relation to the untreated control group: * $P < 0.05$; ** $P < 0.01$.

tistical analysis showed that there were significant differences in comparison with the control group (in both cases $P < 0.01$). On the other hand, this effect was not observed when the HS liposomes were inoculated 10 days after infection. The empty liposomes (without HS) did not produce any curative effect, the counts being similar to those in the untreated control group. It should also be pointed out that, parallel to the reduction in the number of cfu/spleen, the size and weight of these was greater than in the untreated control group: 0.206 g (lipo HS −1) and 0.304 g (lipo HS +2), as opposed to 0.119 g in the control group.

4. Discussion

In recent years new techniques for the production of safe and powerful vaccines have been developed. Nevertheless, the antigens obtained by techniques of fractionation, recombinant DNA and peptide synthesis are limited and do not provide a suitable immune response. Recourse has to be made to classical adjuvants, which are toxic. A possible solution is the use of liposomes as adjuvants to reinforce the immune response to different antigens [22,23]. Given that they are not toxic and are avidly captured by antigen-presenting cells, the use of liposomes could be beneficial to prevent illness in which both humoral and cellular immunity are extremely important, as is the case with brucellosis. We carried out a series of experiments where the effect of the inclusion of an antigenic extract of *Brucella* in liposomes (HS complex) on immunization against murine brucellosis was studied.

The presence of HS extract in the liposomes was shown by biochemical analysis techniques (SDS-PAGE) and serological techniques (agglutination), which demonstrated that the HS antigenic extract was at least partially exposed on the surface of the liposome. Treatment of mice with positively charged HS liposomes one day before infection reduced the number of brucellae in the spleen. Neither free HS, nor HS in negatively charged liposomes, gave this effect. These results confirm those obtained in vitro (Vitas et al., unpublished), according to which the positively charged liposomes interact more efficiently with the monocytes, producing a higher degree of activation. The positively charged liposomes would interact more efficiently with the (negatively charged) cells by electrostatic adsorption, which are then internalised by fusion or by endocytosis. The greater efficiency of positively charged liposomes is not due to a greater incorporation of HS in these vesicles, as analysis of lipopolysaccharide and proteins revealed a similar incorporation in both type of liposomes.

When the effect of the administration of HS liposomes before or after infection was studied, we observed that a protective effect was only produced if the liposomes were inoculated 1 day before or 2 days after infection, but not when administered 10 days after. Administration of HS-positive liposomes 1 day before infection could lead to an activation of monocytes, so that their defence mechanisms increase. Moreover, we should point out that, parallel to the reduction in the number of cfu/spleen, their size and weight was higher than those of the untreated control group. Allen et al. [24] also observed marked splenomegaly in mice treated with liposomes which contained lipid A. We believe that this could be due to a recruitment of polymorphonuclear cells and lymphocytes in the infection zone, although cytology of ultra-thin spleen sections suggests that hematopoiesis also occurred (results not shown).

Finally, we observed a reduction in toxicity of the LPS when these HS extracts were included in positively charged vesicles. The reduction in toxicity of the LPS when included in liposomes was demonstrated by Dijkstra et al. [25]. A direct interaction of lipid A with appropriate plasma membrane components of mononuclear phagocytes is necessary for an efficient biological response. However, this interaction is prevented by the stable insertion of LPS into the liposomal membrane. The greater effect of the cationic liposomes could lie in the fact that at physiological pH they would be expected to interact more efficiently with the negatively charged LPS. The adverse problems [26–28] associated with the use of LPS as a non-specific immunostimulant, or as a specific antigen, could be eliminated by using these liposomes as adjuvants.

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